

09/889015

532 Rec'd PCT/PTO 10 JUL 2001

~~TRANSLATION FROM GERMAN~~~~PATENT APPLICATION~~TitleMycotoxin AdsorbentBackground of InventionDescription

1. Field of Invention

The present invention concerns a mycotoxin adsorbent, especially for adsorption of aflatoxins and other mycotoxins (non-aflatoxins) in feeds, cereals, grains and animal feeds.

2. Prior Art

Background of the Invention

The term mycotoxin encompasses a group of toxic substances that are formed by different naturally occurring fungi. About 300 to 400 mycotoxins are now known. Cereals and grains are generally considered the natural environment for these fungi. Whereas some types of fungi already develop in the spike in the still maturing grain, other types primarily attack grain supplies being stored when a certain minimum moisture and ambient temperature are present.

All so-called mycotoxins have a health-hazardous effect primarily on agricultural animals fed with infected grain, but secondarily on humans as well via the food chain. For example, aflatoxins are responsible for the so-called X-disease of turkeys, which destroyed about 100,000 animals in Great Britain in 1960/61, which had been fed with moldy peanut flour.

Some of the most important mycotoxins are:

Aflatoxins B₁, B₂, G₁, G₂: these are formed by various Aspergillus species. Aflatoxin B₁ is carcinogenic even in microgram amounts and causes stomach and liver damage.

Ochratoxin is formed by Aspergillus ochraceus and Penicillium viridicatum and causes kidney damage.

Zearalenone is formed by Fusarium graminearum, which grows on corn, barley and wheat. It is an estrogen-like substance that causes fertility disorders and is suspected to be carcinogenic.

Fumonisine is formed by fungi of the genus Fusarium and has been implicated, among other things, in horse deaths.

T2 toxins and T2-like toxins (trichothecenes) are formed by fungi of the genus Fusarium.

Moreover, there are a number of additional mycotoxins, like deoxynivalenol, diacetoxyscirpenol, patulin, citrinine, byssochlamic acid, ochratoxin, sterigmatocystine, moniliformine, ergot alkaloids, ergochrome, cytochalasane, penicillinic acid, zearalenone, rubratoxins, trichothecenes (cf. Römpps, Chemie-Lexikon, 8th Edition, 1985, page 2888), and others, which occur, ~~however,~~ ^{only in isolated fashion} in concentrations that cause health problems in feeds. *Only in isolated circumstances,*

Several different toxins that ~~were~~ ^{can be discovered} recognized as causal agents of health problems in humans and animals ~~could be determined~~ ^{utilization} in different feeds by the ~~development~~ ^{have been} of more sensitive analysis methods. A number of studies ~~were~~ able to demonstrate that several toxins can occur simultaneously in feeds. This simultaneous occurrence can significantly influence the toxicity of the mycotoxins. In addition to acute damage to agricultural animals that receive mycotoxin-contaminated feed, health impairment in humans ~~is also being~~ ^{has been} discussed in the literature, ~~which~~ ^{after even} such impairment develops by long-term intake of foods weakly contaminated with mycotoxins.

In a recent study of suspected feed samples, aflatoxin, deoxynivalenone or fumonisine were found in more than 70% of the investigated samples (cf. "Understanding and Coping with Effects of Mycotoxins in ~~live~~ Dog Feed and Forage", North Carolina Cooperative Extension Service, North Carolina State University; <http://www.ces.ncsu.edu/drought/dro-29.html>).

In many cases, the economic effects relative to reduced productivity of the animals, increased occurrence of disease by immune suppression, damage to vital organs and an adverse effect on ~~ever~~ reproductive^{ity} are greater than the effects caused by death of the animals by mycotoxin intoxication.

A ^{can be adsorbed} ~~is fixed~~ by ^{others} ~~some minimal~~ absorbents, like zeolite, bentonite, aluminum silicate and ~~other~~, because of their specific molecular structure (cf. A. J. Ramos, J. Fink-Gremmels, E. Hernandez, "Prevention of Toxic Effects of Mycotoxins by Means of Non-nutritive Adsorbent Compounds", J. of Food Protection, Vol. 59(6), 1996, page 631-641). However, this is not true for most other mycotoxins. An attempt has been made to expand ^{for} the adsorption capacity of mineral adsorbents ~~to~~ non-aflatoxins as well.

A dry particulate animal feed additive is described in WO 91/13555, which contains phyllosilicate particles that are coated with a sequestering agent. An increase in sorption rate can be achieved by ~~this~~ ^{process} but complete (> 90%) elimination of the introduced toxins cannot be achieved. Good results are described in the prior art with ion exchange resins or high-quality activated carbon, but such solutions are not ~~relevant to practice~~ ^{PRACTICAL} for cost reasons.

Organophilic clays are used, among other things, in the prior art, to treat liquid wastes with organic contaminants, in order to solidify them and facilitate their disposal (cf. EP-0,560,423).

S. L. Lemke, P. G. Grant and T. D. Phillips describe in "Adsorption of Zearalenone by Organophilic Montmorillonite Clay", J. Agric. Food Chem. (1998), pages 3787-3796 an organically modified acid montmorillonite clay, which is capable of adsorbing zearalenone. The best adsorption rates were exhibited by clays that were exchanged with cations containing C₁₆ alkyl groups, namely, hexadecyltrimethylammonium (HDTMA) and cetylpyridinium (CP). Noticeable adsorption rates were only achieved from a coating ^{with a cation exchange capacity (CEC)} of more than about 75% of the cation exchange capacity (CEC).

The use of organically modified clay for adsorption of fumonisine B1 is described in Lemke, S. L., Ottinger, S. E. and Phillips, T. D., Book of Abstracts, 216th ACS National Meeting, Boston,

1998. Quaternary ammonium compounds having a C₁₆ alkyl group are used for organophilization.

The task of the present invention is to prepare an adsorbent based on layered silicates (phyllosilicates) that adsorbs not only aflatoxins, but also other important mycotoxins (non-aflatoxins) with high efficiency and, at the same time, is ~~so~~ cost-effective that it can be used in practice. The adsorbent ~~is also supposed to exhibit~~ ^{exhibits} stable adsorption of mycotoxins under physiological conditions, as occur, for example, after absorption with the feeds in the digestive tract of animals.

Summary of Invention

It was surprisingly found that, by appropriate modification of a layered silicate or part of it, mycotoxin adsorbents can be produced that can effectively adsorb both aflatoxins and non-aflatoxins, like zearalenone, ochratoxin, deoxynivalenone, T2 toxins or fumonisin, ^{which} ~~and~~ are also cost-effective.

Detailed Description of Preferred Embodiment

According to a first aspect of the invention, by modification of a layered silicate with a quaternary onium compound with a long-chain C₁₀ to C₂₂ alkyl group and at least one aromatic substituent, ^{even} ~~and also with relatively limited amounts thereof,~~ a significant increase in adsorption performance of such a material for mycotoxins can ~~already~~ be achieved.

The layered silicates listed in Ullmann's Encyclopedia of Industrial Chemistry, Vol. 21, pages 370-375 (1982) fall under the layered silicates employable ^{as} in the adsorbent according to the invention. In particular, the activatable natural and synthetic clay minerals, like smectites, including montmorillonite, beidellite, nontronite, volkonskoite, stevensite, hectorite, swinefordite, saponite and saucinite, the vermiculites, illites, mixed layer minerals, palygorskite (attapulgite) and sepiolite, can be used. The two last-named minerals are also called hormites.

According to a preferred variant of the invention, the layered silicate is a three-layered silicate, for example, a naturally occurring smectitic clay, especially a bentonite clay. Swellable layered silicates with a relatively high swelling volume are preferred, in particular, like calcium bentonites with a swelling volume of about 10 mL/g or more, or layered silicates converted by

ion exchange to the Na^+ form with a swelling volume of about 20 mL/g or more. It is assumed that the specific adsorption performance is positively influenced by ~~the~~ high swellability. However, acid-activated bentonite can also be used.

It was found that very good adsorption performance ~~is~~ ^{can be} achieved for mycotoxins ~~already~~ ^{even} at an exchange rate ~~being~~ well below 75% of the cation exchange capacity (CEC) of the layered silicate. Even ~~during~~ ^{when the rate is as low as} exchange of 2 to 30%, preferably 2 to 15%, especially 2 to 10%, of the CEC, the adsorbents according to the invention exhibit significant adsorption ~~performance~~ for mycotoxins.

According to a preferred variant, for example, a bentonite with a cation exchange capacity from 5 to 100 meq/100 g can be uniformly coated with ~~an amount~~ of onium ions corresponding to about 3 to 15 meq/100 g.

Quaternary ammonium compounds and pyridinium compounds can be used, in particular, as quaternary onium compounds. ~~With the stipulation that~~ ^{If} the quaternary onium compounds contain (at least) a long chain C_{10} to C_{22} alkyl group and at least one aromatic substituent. All onium compounds suitable for organic modification of layered silicates that are known to one skilled in this field can be used. The quaternary onium compounds can also contain an aralkyl substituent (as aromatic substituent).

According to a preferred variant, stearyl (tallow)-benzyldimethylammonium chloride (C_{16} - C_{18} DMBA) is used as quaternary ammonium compound. Additional preferred onium compounds are:

- Coconut alkyldimethylbenzylammonium chloride (C_{12} - C_{16} DMBA)
- Dimethyllaurylbenzylammonium chloride (C_{12} - C_{14} DMBA)
- Distearyl methylbenzylammonium chloride (C_{16} - C_{18} DMBA)
- Quaternized tallow imidazolinium methosulfate.

The quaternary onium compounds can be used either directly or formed in situ during activation of the layered silicate by combined use of secondary and tertiary amines.

It is assumed that the aromatic group(s) and the long chain alkyl group of the quaternary onium compound cooperate to achieve the advantageous adsorption performance. Without restricting the present invention to a theoretical mechanism, it is assumed that the vicinal or isolated carbonyl groups present in nearly all mycotoxins ^{interact} ~~participate in interaction~~ with the adsorbents according to the invention.

In addition to improved adsorption performance of mycotoxins, it was also found that the adsorbents according to the invention exhibit an efficient and stable adsorption of mycotoxins during a reduction of the pH values, as occurs, for example, during uptake of feeds in the acid gastric medium of a monogastric animal, or ~~also~~ on transition from an acid to neutral or slightly alkaline pH value, as occurs during passage of the food slurry through the digestive tract.

According to a second aspect of the invention, the mycotoxin adsorbent contains a mixture of an organically modified layered silicate and a ^{Not} ~~non~~ organically modified layered silicate, in which the organically modified layered silicate in the mixture is exchanged at least 75% (referred to the total CEC) with a quaternary onium compound.

The layered silicates used according to this variant of the invention correspond to those mentioned above.

It was found that, according to this variant of the invention, even when quaternary onium compounds containing no aromatic substituents are used, good adsorption performance for mycotoxins can be achieved. In principle, all onium compounds suitable for organic modification of layered silicates that are known to one skilled in this field can be used. However, those quaternary ammonium compounds that have (at least) one long chain C₁₀-C₂₂ alkyl group and preferably at least one aromatic substituent, as described above, are preferred.

Generally, the mixture will contain about 0.1 to 50 wt.%, especially about 0.5 to 20 wt.%, of organically modified layered silicate. It was surprisingly found that, even at a fraction of more than about 2 wt.% organically modified layered silicate in the mixture, almost complete (more than 90%) adsorption of mycotoxins (aflatoxins and non-aflatoxins) occurs even at acid pH

values. It is therefore assumed, without restricting the invention to a theoretical mechanism, that the hydrophobic surface of the organically modified layered silicate and the surface of the unmodified layered silicate interact during effective adsorption and slight desorption of mycotoxins. For example, it is assumed that the aflatoxins primarily bind to the unmodified layered silicate in a mixture of organically modified and unmodified layered silicate, so that the surface of the organically modified layered silicate is available for adsorption of the non-aflatoxins that cannot be adsorbed on the unmodified layered silicate. Good adsorption performance with respect to non-aflatoxins is therefore also guaranteed at relatively high aflatoxin concentrations.

Since the organically modified layered silicate represents the ~~essential cost factor~~^{most costly portion} of the mixture, the smallest possible fraction of organically modified layered silicate is chosen in the mixture under practical conditions, but one in which good adsorption performance is observed. The optimal fraction of organically modified or unmodified layered silicate can be determined in individual cases by one skilled in the art by means of a routine experiment.

According to a preferred variant, however, generally about 0.5 to 30 wt.%, especially to 15 wt.%, ~~most~~^{most} especially to 10 wt.%, of organically modified layered silicate is used in the mixture.

Adsorption of mycotoxins in an aqueous solution remains stable even during a reduction of the pH value or a transition from acid to neutral or slightly alkaline pH, as occurs under physiological conditions during digestion of feed, i.e., the desorption rate is low.

Another advantage of the adsorbent mixture according to the invention is that, because of the relatively limited fraction of organically modified layered silicate in the mixture, desired hydrophobic substances, like lipophilic vitamins or essential fatty acids, are only bound to a limited degree to the adsorbent and are therefore available for resorption in the digestive tract.

The same advantage is obtained during relatively limited exchange in the case of use of a partially organically modified layered silicate.

According to another aspect of the invention, a feed additive that contains the adsorbent according to the invention is prepared.

It is also possible to produce premixes that contain a fairly high percentage of more than about 50% organically modified layered silicate ^{which} are mixed in a second step to produce an adsorbent according to the invention or a feed additive with an unmodified layered silicate.

The mycotoxin adsorbents according to the invention can contain additional components that appear useful for the corresponding application, for example, feed supplements or agents for (enzymatic) detoxification of mycotoxins.

Examples

The cation exchange capacity was determined as follows.

5 g of clay was screened through a 63 µm sieve and dried at 110°C. Precisely 2 g was then weighed out and mixed with 100 mL of 2 N NH₄Cl solution. The suspension was boiled under reflux for an hour. After standing for about 16 hours, the NH₄⁺ clay was filtered off via a membrane suction filter and washed with deionized water (about 800 mL) to ~~remove the freedom from ions~~. Detection of ~~freedom from~~ ^{effective elimination of} ions of the wash water was carried out for NH₄⁺ ions with the Nessler reagent sensitive to this (Merck company). The washed out NH₄⁺ clay was taken up by the filter, dried at 110°C for 2 hours, ground, screened (63 µm sieve) and dried again at 110°C. The NH₄⁺ content of the bentonite was then determined according to Kjeldahl. The CEC of the clay is the NH₄⁺ content of the NH₄⁺ clay determined by the Kjeldahl method. The data are given in meq/100 g of clay.

The invention is now explained by means of the following examples.

The different mycotoxins were acquired as crystalline pure substances (SIGMA AG) and taken up in methanol or acetonitrile (50 µg/mL). To perform the adsorption experiment, dilutions were produced using buffer solutions (dipotassium hydrogen phosphate + citric acid), each of which contained 100 µg of the different toxins per liter.

Example 1

A natural Ca bentonite was used for the adsorption experiment, having a cation exchange capacity of 90 meq/100 g. Complete exchange of the interlayer cation (100% of the CEC) occurred according to the prior art (S. L. Lemke, P. G. Grant and T. D. Phillips, "Adsorption of Zearalenone by Organophilic Montmorillonite Clay", J. Agric. Food Chem. (1988), page 3790) with the following quaternary ammonium ions:

CP:	Cetylpyridinium chloride
HDTMA:	Hexadecyltrimethylammonium chloride
SBDMA:	Stearylbenzyldimethylammonium chloride
ODDBMA:	Octadecyldibenzylmethylammonium chloride

The organophilized bentonites were dried and finely ground, so that the residue on a 90 µm sieve was less than 10%. They were then added in an amount of 0.02 wt.% to mycotoxin-containing aqueous solutions (100 mL), each of which contained 100 µg of the three mycotoxins aflatoxin B1, ochratoxin A and zearalenone in 1 L of aqueous solution (pH 7).

The suspensions so produced were shaken at room temperature for 1 hour upside down, and then centrifuged for 5 minutes at 1500 rpm. The clear supernatant was extracted with 2 mL hexane and the hexane phase investigated by HPLC for the amounts of toxins remaining in the solution.

HPLC determination occurred under the following conditions:

Column: Spherisorb ODS-2 125 × 4 mm

Mobile phase:

Aflatoxin: 600 mL of a 1 mmol NaCl solution/200 mL acetonitrile/
200 mL methanol

Ochratoxin: 570 mL acetonitrile/410 mL water/20 mL acetic acid

Zearalenone: 570 mL acetonitrile/410 mL water/20 mL acetic acid

Flow rate: 1.5 mL/min

Detector: Fluorescence
 Wavelength: EX 365 nm / EM 455 nm
 Furnace temperature: 30°C (aflatoxin, ochratoxin);
 40°C (zearalenone).

The percentage adsorption rates were calculated by means of the results. The obtained results are summarized in Table 1.

Table I

Effect of onium ion of different organoclays on adsorption of mycotoxins

	Aflatoxin B1 Adsorption [%]	Zearalenon Adsorption [%]	Ochratoxin Adsorption [%]
100 % CP-Organoton	65,4	43,5	38,7
100 % HDTMA-Organoton	78,2	45,8	46,1
100 % SBDMA-Organoton	88	78,3	82,5
100 % ODDBMA-Organoton	86,5	82,8	85,4

all #s w/
periods

Zearalenon = Zearalenone

Organoton = Organoclay

It is apparent from Table I that the mycotoxin adsorbents according to the invention adsorbed both aflatoxins and non-aflatoxins much better than the CP and HDTMA organoclays according to the prior art.

Example 2

The bentonites modified with CP, HDTMA or SBDMA, produced as described in Example 1, were mixed with natural unmodified Ca bentonite (cf. Example 1 above) with comparable particle fineness in the following ratio: 96 wt.% Ca bentonite + 4 wt.% organoclay.

The organophilized bentonites were added to mycotoxin-containing aqueous solutions (100 mL) in an amount of 0.5 wt.%, each of the solutions containing 100 µg of the three mycotoxins aflatoxin B1, ochratoxin A and zearalenone in 1 L of aqueous solution (pH 7).

The suspensions so produced were shaken upside down at room temperature for 1 hour, and then centrifuged for 5 minutes at 1500 rpm. The clear supernatant was extracted with 2 mL hexane and the hexane phase investigated, as in Example 1, by HPLC.

The percentage absorption rates were calculated by means of the results. The obtained results are summarized in Table 2.

Table II

Effect of onium ion of different organoclays in clay mixtures on adsorption of mycotoxins

	Aflatoxin B1 Adsorption [%]	Zearalenon Adsorption [%]	Ochratoxin Adsorption [%]
100 % Ca-Bentonit	90,1	18,3	11,8
96 % Ca-Bentonit + 4 % CP-Organoton	90,3	61,3	57,6
+ 4 % HDTMA-Organoton	89,2	62,4	65,7
+ 4 % SBDMA-Organoton	90,6	90,4	93,2

with numbers
with periods

Zearalenon = Zearalenone

Bentonit = Bentonite

Organoton = Organoclay

It is apparent from Table II that the mycotoxin adsorbent according to the invention, which contains a mixture of unmodified bentonite with SBDMA organically modified bentonite almost fully adsorbed both aflatoxins and the non-aflatoxins in contrast to the adsorbents according to the prior art.

Example 3

A bentonite modified with SBDMA, produced as described in Example 1, was mixed with natural unmodified Ca bentonite with comparable grain fineness in the weight ratios listed in the following Table III.

The mixtures so obtained were added in an amount of 0.5 wt.% to mycotoxin-containing aqueous solutions (100 mL), each of which contained 100 µg of the three mycotoxins aflatoxin B1, ochratoxin A and zearalenone in 1 L of aqueous solution at pH 3 or pH 7.

The suspensions so produced were shaken upside at room temperature for 1 hour, and then centrifuged for 5 minutes at 1500 rpm. The clear supernatant was extracted with 2 mL hexane and the hexane phase investigated, as described in Example 1, by HPLC.

The percentage adsorption rates were calculated by means of the results. The obtained results are summarized in Table III.

Table III

Mycotoxin adsorption on mixtures of unmodified and organically modified bentonite

Anteil SBDMA-Organoclay in Ca-Bentonit	Aflatoxin B1 Adsorption [%]		Zearalenone Adsorption [%]		Ochratoxin Adsorption [%]	
	pH 7	pH 3	pH 7	pH 3	pH 7	pH 3
0%	90,1	96,1	18,3	29,5	11,8	19,2
2%	92,1	95,4	82	89,8	79,4	84,8
3%	90	96,3	88,9	92,3	90,7	88,5
4%	90,6	96	90,4	91,7	93,2	90,2
6%	91,9	95,8	90,8	93,4	95,5	90,5

all
numbers
with
periods

Zearalenon = Zearalenone

Left: Percentage of SBDMA organoclay in Ca bentonite

It is apparent from Table III that a very good adsorption of even the non-aflatoxins could be achieved with just 2 wt.% SBDMA organoclay in the mixture.

Example 4

An organophilized SBDMA bentonite was produced generally as described in Example 1, less SBDMA being used for modification, in order to achieve uniform exchange at a level of 8% of the CEC of bentonite.

An SBDMA bentonite exchanged to 100% of the CEC, produced as described in Example 1, was also mixed with natural unmodified Ca bentonite with comparable particle fineness in a ratio of 96 wt.% Ca bentonite + 4 wt.% SBDMA organoclay.

500 mg of the different adsorbents were metered into each 100 mL of aqueous toxin solution, which corresponds to an amount of 0.5%, referred to the supplied solution.

The suspensions so produced were shaken upside down at room temperature for 1 hour and then centrifuged for 5 minutes at 1500 rpm. The clear supernatant was extracted with 2 mL hexane and the hexane phase investigated, as described in Example 1, by HPLC.

For the desorption experiments, the solid obtained after centrifuging and separation of the liquid phase was resuspended in 100 mL of a fresh buffer solution with the desired pH value, the suspension shaken upside down at room temperature for 1 hour and treated further as described above.

Table IV

Adsorption/desorption behavior and its influencing by the pH value of the medium

	Adsorption / Desorption an SBDMA-Organoton. belegt mit 8% der CEC	Adsorption / Desorption an Gemisch aus Ca-Bentonit + 4 % SBDMA-Organoton
Aflatoxin B1		
Adsorption bei pH 7	>97,5%	>97,5%
Desorption I bei pH 3	< 2,5%	< 2,5%
Desorption II bei pH 7	< 2,5%	< 2,5%
Ochratoxin		
Adsorption bei pH 7	87,5%	93,2%
Desorption I bei pH 3	7,2%	3,2%
Desorption II bei pH 7	5,1%	4,6%

all numbers
with periods

Headings, Left to Right:

Adsorption/desorption on SBDMA organoclay coated with 8% CEC;

Adsorption/desorption on mixture of Ca bentonite + 4% SBDMA organoclay

bei = at

It is apparent from the table that very good adsorption rates were achieved at pH 7 both with the SBDMA organoclay exchange to 8% of the CEC and the mixture of 96% Ca bentonite and 4% SBDMA organoclay.

Only very limited desorption occurred both during the reduction in pH value of the medium to pH 3 and subsequent rise of the pH value again to 7. Because of this, stable adsorption is demonstrated on the organoclays and organoclay mixtures according to the invention.

For the nonorganically modified bentonite, the adsorption rate was < 20% for ochratoxin and the overall desorption (I + II) was > 40%. When fully exchanged SBDMA organoclay was used, complete adsorption > 97.5% was achieved for aflatoxin and ochratoxin, the desorptions (I, II) were < 2.5%.